EXHIBIT GG

Human plasma α_2-macroglobulin promotes *in vitro* oxidative stress cracking of Pellethane 2363-80A: *In vivo* and *in vitro* correlations

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It is hypothesized in this study that the phenomenon of environmental stress cracking (ESC) in polyetherurethane is caused by a synergistic action of biological components in the body fluids, oxidative agents, and stress. An *in vitro* system is designed to mimic the *in vivo* system; human plasma contains certain biological components that can act as a stress cracking promoter, while H_2O_2 (Co) solution provides an oxidative reaction comparable to that observed in the respiratory burst of adherent macrophages and foreign-body giant cells. It is demonstrated that the phenomenon of *in vivo* stress cracking in Pellethane 2363-80A is duplicated by an *in vitro* system that involves a pretreatment of prestressed specimens with human plasma at 37°C for 7 days followed by oxidation in 10% hydrogen peroxide with 0.10*M* cobalt chloride at 50°C for 10 days. The pretreatment with plasma has a synergistic effect with the oxidation by H_2O_2 (Co) treatment to produce ESC. A plasma component responsible for promoting stress cracking in Pellethane polyurethane is identified to be α_2-macroglobulin (α_2M). © 1993 John Wiley & Sons, Inc.

INTRODUCTION

With their unique and excellent physical, mechanical, and biocompatible properties, polyetherurethane elastomers have been used as constituent materials of many medical devices. Since these medical devices are often intended to perform reliably and safely while implanted in the body for prolonged periods of time, their long-term biostability is of vital importance to ensure safety and effectiveness when in contact with the tissue or body fluids of the living body. In certain limited circumstances, unintended biodegradation and surface cracking of polyurethanes have created increasing needs in biomaterials research to understand further the nature of biodegradation/biostability of the materials.[1,2]

In vivo environmental stress cracking (ESC) in polyetherurethanes is believed to be a complex phenomenon. It is related to the chemical property of polyurethanes (e.g., polyether content),[3–5] stress state of the material in use (e.g., design manufacturing processes),[1,6] cell or tissue/polymer interactions (e.g., macrophage adhesion and foreign-body giant cell formation),[7] and the body's physiological environment (i.e., so far unknown plasticizing or crack propagating agent).[1,3,8] The mechanism for the ESC is still not clear due to unsuccessful duplication of this phenomenon by an *in vitro* method.[3,6,8]

In this study, the investigation was conducted under a hypothesis that biological components in the body fluids, oxidative agents, and stress or strain act synergistically to produce the stress cracking of polyurethanes. An *in vitro* system that duplicates the ESC observed with polyetherurethane *in vivo* was

TABLE I
Biological Fluids Used for *In Vitro* Treatment of Pellethane 2363-80A

Human blood plasma (citrated)
Plasma PEG fraction I
Plasma PEG fraction II
Plasma PEG fraction III
α_2-Macroglobulin (2 mg/mL)
Ceruloplasmin (1 mg/mL)
Transferrin (2.3 mg/mL)
Lipoprotein (0.54 mg/mL)

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developed. Biological component(s) involved in ESC were identified. The relationships among biological components, stress state, and oxidation in PEU biodegradation are also discussed.

MATERIALS AND METHODS

Prestressed polyurethane specimens and different treatments

The poly(etherurethane) (PEU) elastomer used in this study was Pellethane 2363–80A. The base polymer was prepared from 4,4′-diphenyl bis(phenylisocyanate) (MDI) and poly(tetramethylene glycol) (M_w = 1000) (PTMEG) and chain extended with 1,4-butanediol. The polymer had a molecular weight of 95,000 relative to polystyrene. The bulk material contained two additives: a phenolic antioxidant and a bis(stearamide wax) processing agent.

The prestressed Pellethane 2363-80A tubing specimens (2 mm in diameter and 12.5 mm in length) were obtained from Medtronic, Inc. The polymer tubing was soaked in acetone for 1 h to extract the additives, prior to being stretched to 400% elongation over a mandrel. A detailed description of the specimen preparation is provided elsewhere.[7] This type of prestressed specimen is known to undergo rapid biodegradation and stress cracking *in vivo* based on our previous study.[7] For comparison, the 5- and 10-week implants in rats from the previous study were analyzed to correlate with the *in vitro* results in this study.

In the *in vitro* treatment, the prestressed specimens were immersed in the biological fluids at 37°C for 7 days, rinsed in distilled water, and put into an oxidizing solution at 50°C for 9 or 10 days. The oxidizing agent was an aqueous solution of 10% hydrogen peroxide (Fisher Scientific) and 0.10*M* cobalt chloride. The proposed role of cobalt chloride was to facilitate the decomposition of hydrogen peroxide to produce oxygen radical species.[9,10] During the treatment, the peroxide/cobalt chloride solution was changed with fresh solution every 3 days. The biological fluids were human blood plasma, fractionated plasma, and simple protein solutions (Sigma), respectively (Table I). After the treatment, the polymer specimens were rinsed in distilled water, dried in air, and then stored in a vacuum desiccator for scanning electron microscopy (SEM, JOEL 840), ATR-FTIR, and GPC analyses.

The procedure for plasma fractionation with polyethylene glycol (PEG) was adapted from the method described by Hao et al.[11] All procedures were performed at 4°C. A beaker containing 100 mL citrated human plasma was placed in an ice bath. Solid PEG (M_w 4000, Polysciences, Inc.) was added slowly with stirring to plasma in the amount of 10 g/100 mL. After 60 min, the 10% PEG precipitate, designated fraction I, was removed by centrifugation for 30 min at 2000 rpm. An additional 10 g of PEG was added to the supernatant followed by centrifugation to obtain fraction II (the 10–20% PEG precipitate) and fraction III (the supernatant containing 20% PEG). The precipitates were reconstituted to the original volume of plasma (100 mL) with phosphate-buffered saline (PBS). The distribution of proteins in the three fractions is shown in Table II.[11] Fraction I is rich in fibrinogen, plasminogen, C-3 component of complement, IgG, and β-lipoproteins. Fraction II is rich in α_2-macroglobulin (α_2-M), IgA, prothrombin, and other coagulation factors (prothrombin complex). Fraction III contains mainly albumin, α_1-acid glycoprotein, transferrin, and 20% PEG.

ATR-FTIR and GPC analyses

To obtain ATR-IR spectra, the prestressed tubing specimens were opened by an incision along the tubing direction and the mandrels were removed. The ATR-IR spectra were obtained on the outer surface of the opened tubes with a Nicolet 800 spectrometer using the micro-ATR attachment made by Harrick Scientific, Inc., that featured two 4× beam condensers and continuous beam angle variation. To obtain degradation depth profiles, internal reflection elements (IRE) of Germanium with 60°, 45°, and 30° endface angles, and KRS-5 with 60° and 45° endface angles were used and combined with different incident angles of the IR beam.

The instrument employed for GPC analysis was a Varian DS-651 LC Star System with a 9010 Solvent Delivery System, a 9065 Polychrom UV diode-array detector, and a RI-4 refractive index detector set at 40°C. The running eluent was HPLC-grade THF with a flow rate of 1.0 mL/min. The standards and columns were obtained from Polymer Laboratories, Inc. (Amherst, MA). One set of calibration standards were used to calibrate a series of PL-Gel columns of 500, 10,000, and 100,000 Å pore size. Polystyrene calibration standards ranged from 1000 to 2,000,000 molecular weight, with a polydispersity (PDI) of 1.1. Reported molecular weight values were based on the two sets of calibration standards. Deconvolution of the GPC chromatograms was accomplished by using a spectral curve-fitting software (PeakFit 1.0, Jandel); two math functions, gaussian and exponentially modified gaussian, were employed.

Analysis of adsorbed plasma proteins

The adsorption and extraction of plasma proteins was performed on the original, unstrained Pellethane tubing (2 mm in diameter) material. Fifty milligrams

TABLE II
Distribution of Selected Plasma Proteins in PEG Fractions (Ref. 11)

Plasma Proteins	Amount (mg/dL)	Fraction I wt% (mg/dL)	Fraction II wt% (mg/dL)	Fraction III wt% (mg/dL)
Albumin	3440	6 (206)	4 (138)	86 (2958)
IgG	740	88 (651)	15 (111)	1 (7)
Transferrin	223	6 (13)	22 (49)	58 (129)
α_2-Macroglobulin	189	35 (66)	65 (123)	—
Fibrinogen	179	88 (158)	—	—
IgA	157	34 (53)	58 (91)	20 (31)
Haptoglobin	108	2 (2)	40 (43)	56 (61)
α-Lipoprotein	64	15 (10)	25 (16)	50 (32)
C-3 Component	56	93 (52)	7 (4)	—
α_1-Acid glycoprotein	54	—	—	100 (54)
Ceruloplasmin	22	14 (3)	23 (5)	73 (16)
Plasminogen	16	69 (11)	19 (3)	—
β-Lipoprotein	16	100 (16)	—	—
Prothrombin	8	25 (2)	50 (4)	25 (2)
C-1 Esterase inhib.	4	—	—	100 (4)

of the polymer tubing was cut in half longitudinally to avoid entrapment of fluid while immersed in 10 mL of citrated plasma at 37°C for 7 days. After plasma treatment, the material was placed in a clean test tube and rinsed three times with PBS. The tubing material was then cut into small pieces that were transferred into a clean microtube. The adsorbed proteins were extracted in 0.25 mL of 1% sodium dodecyl sulfate (SDS) or the sample buffer used in SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)[12] at room temperature for 24 h. Proteins in the extract were separated by SDS-PAGE using 7.5% or 4–15% acrylamide gradient gels. Separated proteins were visualized by silver stain (BioRad).

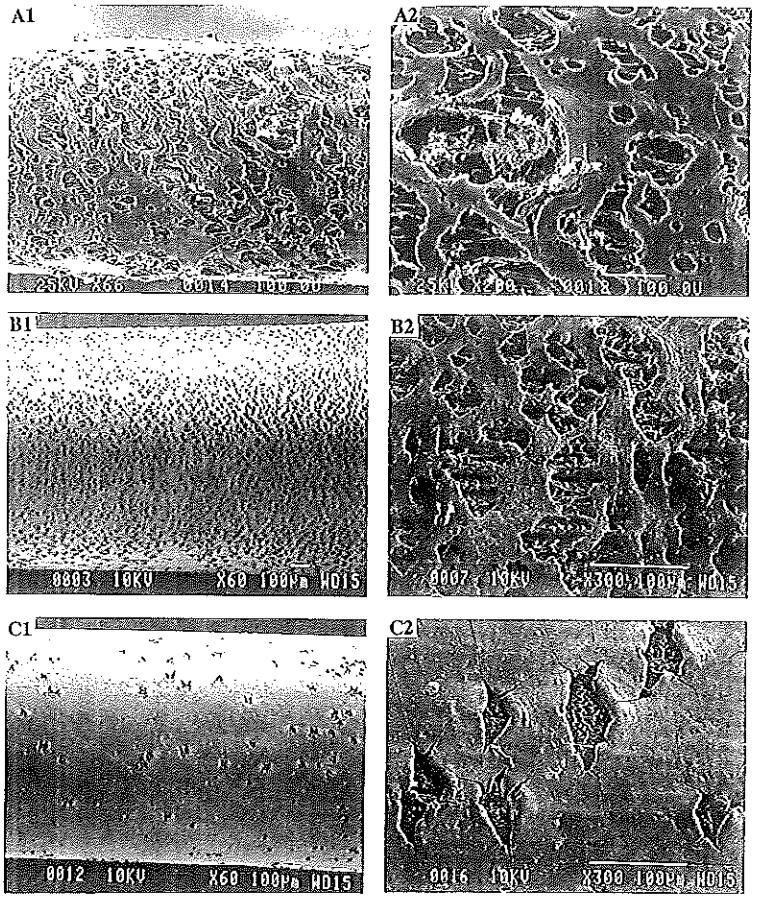

Figure 1. SEM pictures of prestressed specimens after treatments: (A1 and A2) 35 days (5 weeks) implanted; (B1 and B2) 7 day plasma plus 10-day peroxide/cobalt treated; (C1 and C2) 10-day peroxide/cobalt treated.

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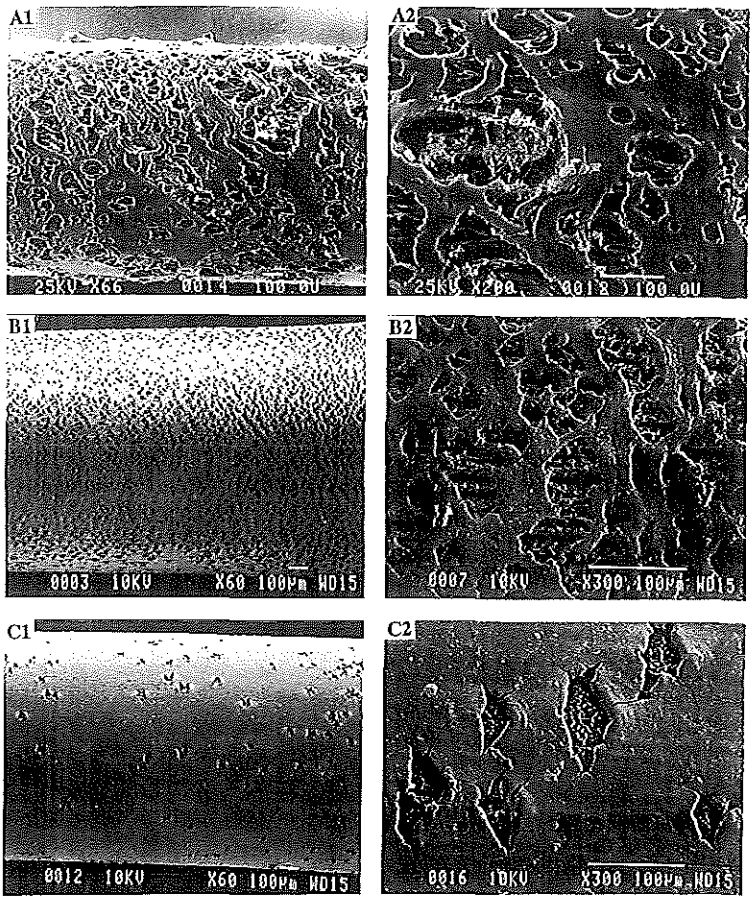

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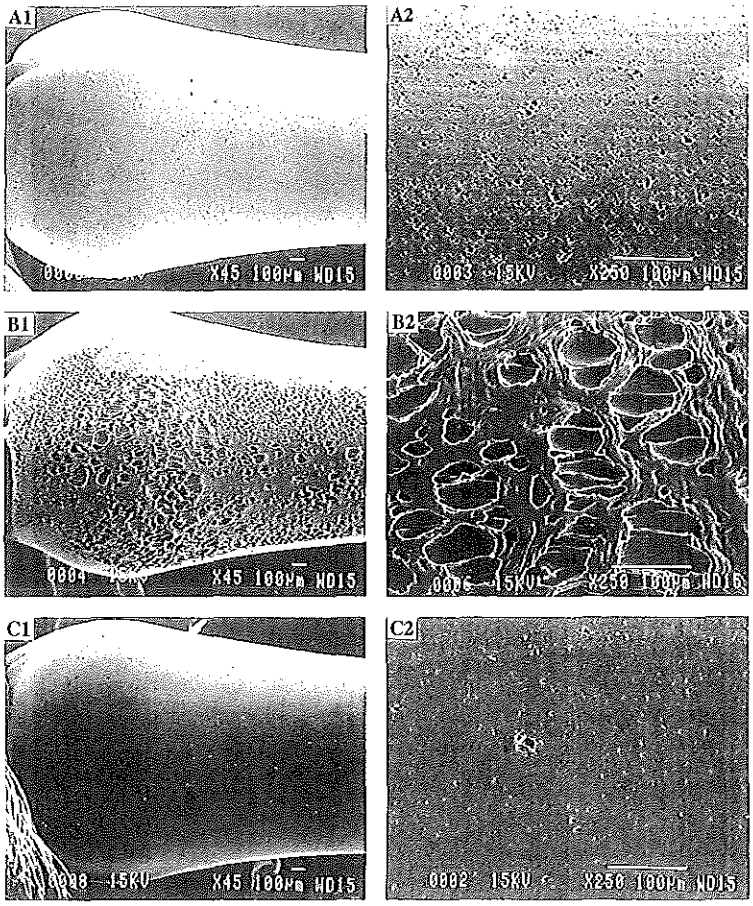


Figure 2. SEM pictures of prestressed specimens after *in vitro* treatments: (A1 and A2) 7 day fraction I plus 9-day peroxide/cobalt treated; (B1 and B2) 7-day fraction II plus 9-day peroxide/cobalt treated; (C1 and C2) 7-day fraction III plus 9-day peroxide/cobalt treated.

The identities of selected proteins were confirmed by immunoblot analysis (Western blot). Briefly, proteins separated by SDS-PAGE were transferred to nitrocellulose (Mini-Transblot, BioRad) for 1 h at 100 V. Nitrocellulose strips were blocked with 5% nonfat dry milk in Tris-buffered saline for 2 h at 25°C prior to application of rabbit primary antibodies to human α_2-macroglobulin, ceruloplasmin, albumin, or α-fetoprotein (nonspecific control) diluted 1:100 in blocking buffer. After 18 h at 25°C, the strips were washed, incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (BioRad) for 2 h, washed again, and developed using an alkaline phosphatase substrate kit (BioRad).

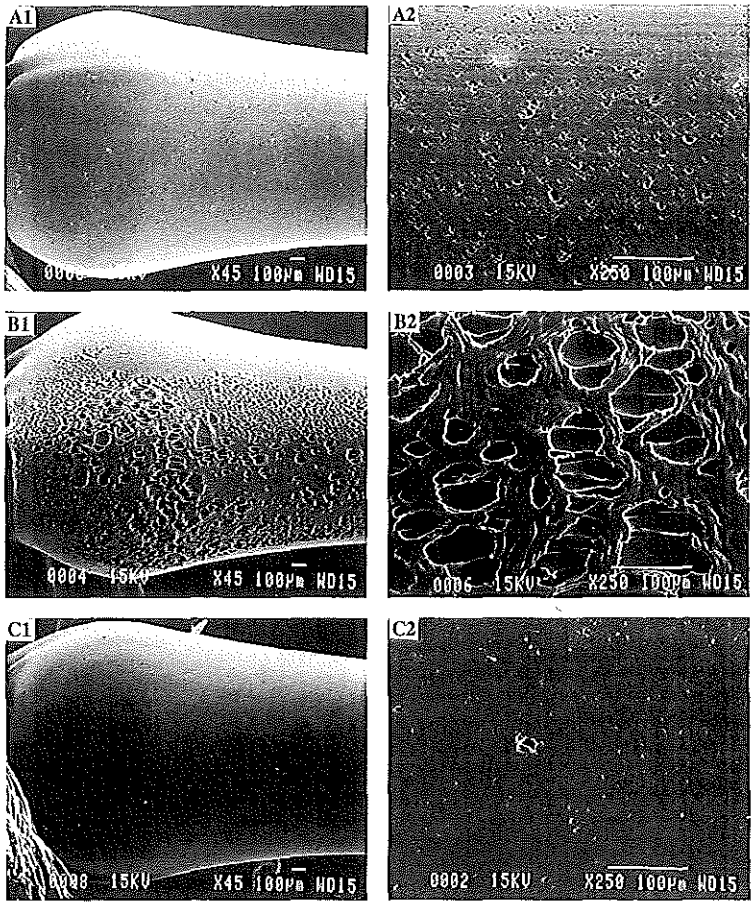
RESULTS

SEM surface examination

After 35 days (5 weeks) implantation, severe cracking on the prestressed (strained) Pellethane 80A was observed under SEM [Fig. 1 (A1), (A2)]. The cracking pattern consisted of independent and interconnecting open cracks. The cracks with a fibrillar structure propagated across the tubing surface along the transverse direction of the applied stress. Similar cracking patterns were found on the *in vitro* specimens after the prestressed samples were first treated with plasma at 37°C for 7 days, and then with 10% H_2O_2 (Co) at 50°C for 10 days [Fig. 1 (B1), (B2)]. For the samples treated only in 10% H_2O_2 (Co) solution at 50°C for 10 days, isolated open cracks and brittle microcracking were observed [Fig. 1 (C1), (C2)].

Figure 2 shows SEM pictures of the prestressed specimens respectively pretreated with plasma fractions I, II, and III, then followed by H_2O_2 (Co) treatment. The pretreatment with fraction II [Fig. 2 (B1, (B2)] produced more severe surface cracking on the specimens than the pretreatment with fraction I or III. The cracking on the specimens treated with fraction II plus H_2O_2 (Co) resembled the patterns observed on the 35-day implant [Fig. 1;(A1), (A2)] and the specimens treated with plasma plus H_2O_2 (Co), with large cracks interconnecting and propagating across the surface, whereas the specimens pretreated with fractions I and II showed micro-pitting with no crack propagation.

In Figure 3, the specimens pretreated with α_2-macroglobulin, α_2-M [Fig. 3(A1), (A2)] and cerulo-



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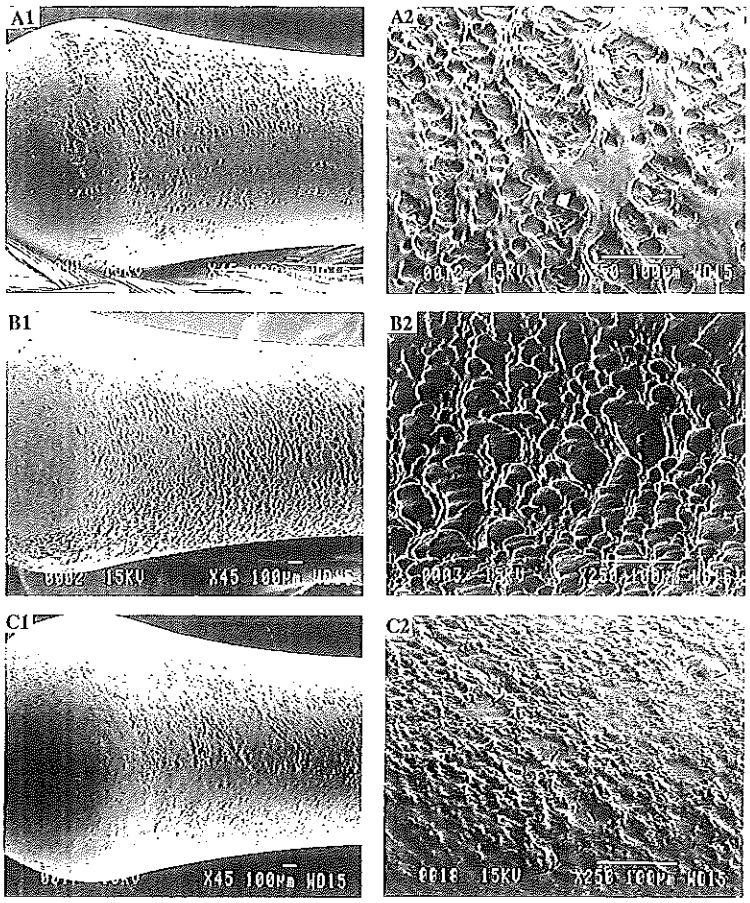

Figure 3. SEM pictures of prestressed specimens after *in vitro*: (A1 and A2) 7-day α_2-macroglobulin plus 9-day peroxide/cobalt treated; (B1 and B2) 7-day ceruloplasmin plus 9-day peroxide/cobalt treated; (C1 and C2) 7-day lipoprotein plus 9-day peroxide/cobalt treated.

plasmin [Fig. 3(B1), (B2)] solutions showed cracking patterns similar to the specimens pretreated with fraction II [Fig. 2(B1), (B2)]. Surface pitting, roughening, and brittle cracking were observed on the specimens pretreated with lipoprotein [Fig. 3(C1), (C2)]. Similar brittle cracking was also found on the specimens treated with transferrin solution plus H_2O_2 (Co).

ATR-FTIR and GPC analyses

Figure 4 shows the ATR-FTIR spectra obtained from the specimens before and after different treatments. Among the samples, the 7-day plasma-treated samples showed essentially the same spectral features as the untreated samples. Samples treated with H_2O_2 (Co) and plasma plus H_2O_2 (Co), as well as the 70-day (10-week) implant, similarly displayed dramatic changes in their spectra, with large decreases in the soft-segment bands (e.g., 1110 cm^{-1} and 2795 cm^{-1}) and the non-hydrogen-bonded urethane carbonyl band (1730 cm^{-1}). In addition to the decrease in some bands several new bands, were also observed [Fig. 4(A), (B)]. By subtracting the spectra of the treated from the untreated samples, more new bands were revealed. Table III summarizes the new bands that appeared in the subtracted spectra of the treated samples in the region of 1800–900 cm^{-1}. The new bands were tentatively assigned to the formation of acid, ester, alkene, and aldehyde groups. To support these assignments, bands from some model compounds are listed in Table III.

The experimental and deconvoluted GPC chromatograms for the untreated and treated specimens are shown in Figure 5. By deconvolution two peaks at 15.2 and 18.0 min were revealed in the chromatograms of the untreated and plasma treated bulk specimens [Fig. 5(A), (B)]. For the specimens treated with H_2O_2 (Co) and plasma plus H_2O_2 (Co), the GPC chromatograms showed that, in addition to the two GPC peaks in the untreated, a small peak existed at 23.0–23.5 min [Fig. 5(C), (D)]. Figure 6 depicts the GPC chromatograms of the specimens implanted for 70 days (10 weeks). The *in vivo* specimen had the same peaks found in the untreated polymer, with a small shoulder at 23.0–23.5 min.

The relative percentages of the first, second, and third GPC peaks in the all of the samples are given in Table IV. The percentage of each GPC peak was determined based on the individual peak area compared to the total of all peak areas. The ratios of the

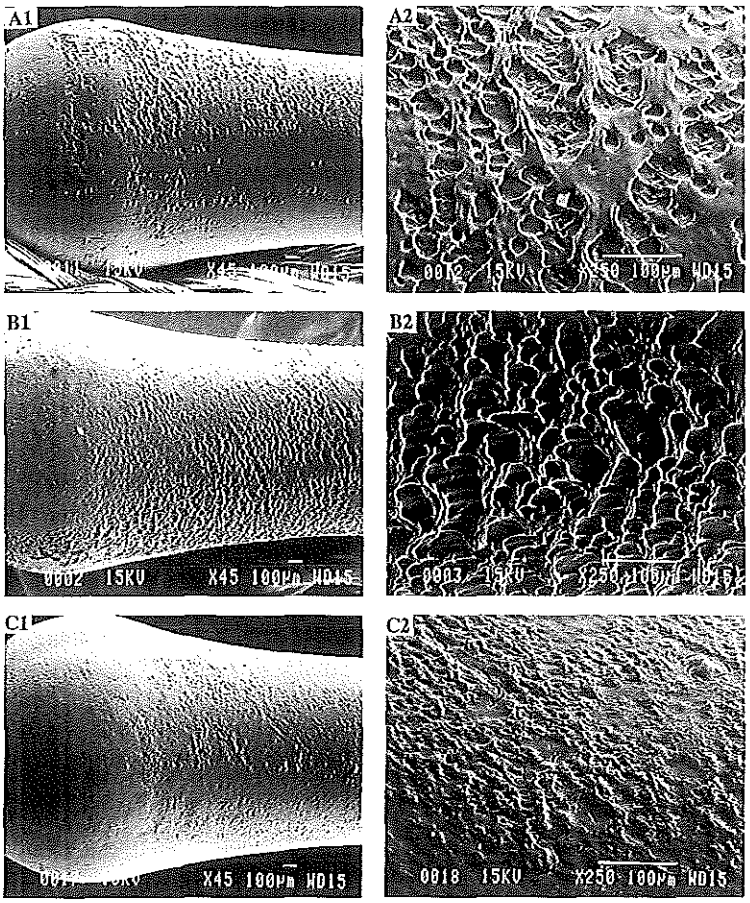

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TABLE III
New Bands in the Subtracted ATR-IR Spectra of Pellethane 2363-80A After *In Vivo* and *In Vitro* Treatments[a,b]

Bands (cm^{-1})	Samples	Intensity	Possible Assignment	Model Compounds[20] Name	Band (cm^{-1})
~1746	IV	Weak	C=O str, sat. aliph. esters;	Ethyl butyrate	1738
	V	Medium		Ethyl caprylate	1739
1683–1630	III	Medium	C=O str, acids with H-bonding	Fumaric acid	1670
	IV	Weak		Butyl vinyl ether	1635
	V	Medium	C=C str, akenes		
1603–1587%	III	Weak		Maleic acid	1589
	IV	Strong	CO_2^- asym. str, acid salts		
	V	Medium			
1402%	III	Strong	C—O str and/or O—H	Butyric acid	1414
	IV	Medium	def, acids CHO in-plane vibr.,	Butyraldehyde	1391
	V	Strong	aldehydes		
1327	III	Weak	C—O str, acids associated O—H def	Succinic acid	1310
	IV	Medium	and C—O str, alcohols C in-plane vibr.,	Butyl vinyl ether	1320
	V	Medium	alkenes		
1285	III	Strong	C—O str and/or O—H def,	Butyric acid	1282
	IV	Strong	acids O—H def and C—O str, alcohols		
	V	Medium			
1230	V	Weak	C—O str, acids associated C—O str, aliph. esters	Butyric acid	1221
				Ethyl caprylate	1256
1174	III	Strong			
	IV	Strong	C—O—C asym. str, esters	Ethyl caprylate	1175
	V	Strong		Poly(ester urethane)[21]	1170
930	III	Strong		Butyric acid	938
	IV	Strong	O—H def, acid	Succinic acid	920
	V	Strong			

Note. [a]str = stretching; def = deformation; aliph. = aliphatic.
[b]III, Treated with H_2O_2 (Co); IV, treated with plasma plus H_2O_2 (Co); V, implanted for 70 days.

first and second peaks for all of the samples were similar. The third GPC peak was the highest in the 7-day plasma plus 10-day H_2O_2 (Co)-treated samples. The 70-day *in vivo* sample was the intermediate value, while the 10-day H_2O_2 (Co)-treated samples had the lowest GPC peak area. The molecular weights and molecular weight distributions for each individual peak are shown in Table V. Decreases in molecular weights, M_n and M_w, of the two major GPC peaks were found for the samples treated with H_2O_2 (Co) or plasma plus H_2O_2 (Co). The third peak molecular weights were higher in the 70-day implant than in the specimens treated with H_2O_2 (Co) or plasma plus H_2O_2 (Co).

Adsorbed plasma protein analysis

Figure 7 shows the protein bands on the SDS-PAGE gel obtained from extracts of plasma-treated Pellethane 2363-80A. The standard (column 1) was a solution of proteins with different molecular weights (Bio-Rad). The extraction in 1% SDS solution gave weaker bands in column 2 due to dilution. More striking bands were seen when the extraction was performed in SDS sample buffer (column 3). The bands from the extracts were compared with bands from commercially obtained purified protein solutions of α_2-macroglobulin, ceruloplasmin, transferrin, and low-density lipoprotein. It was observed that a band ca. 200,000 molecular weight in the extract coincided with a band from α_2-macroglobulin. Immunoblotting (Western) with polyclonal antibody to α_2-macroglobulin indicated a positive band at the same position.

DISCUSSION

In vivo and *in vitro* comparisons

The phenomenon of stress cracking in polyurethanes has been observed in implanted materials or devices.[8,13] One characteristic of this *in vivo* stress cracking is the nature of ductile fracture in the stressed material, with crack propagation and a fibril pull-out type of crack structure. It was shown in the SEM study that oxidizing a prestressed Pellethane 2363-80A specimens in H_2O_2 (Co) solution alone caused isolated cracks and brittle microcracking, but without crack propagation [Fig. 1(C1), (C2)]. However, when the specimens were pretreated with human plasma and then with H_2O_2 (Co) solution [Fig. 1(B1), (B2)], the cracking became ductile with large open cracks

interconnecting and propagating across the surface along the transverse direction of the applied stress (strain) direction. Morphologically, the pattern of *in vitro* stress cracking had remarkable similarity to that of *in vivo* stress cracking [Fig. 1(A1), (A2)].

Chemical analysis by ATR-FTIR revealed similar chemical changes that occurred after the prestressed specimens were subjected to long-term implantation and *in vitro* treatment with H_2O_2 (Co) or plasma plus H_2O_2 (Co). The oxidative degradation is believed to take place mainly in the poly(tetramethylene ether) soft segments with oxygen radicals attacking either α-methylene or β-methylene groups. This causes a decrease in the intensities of soft-segment bands. Although the attack at the β position is less probable than at the α position, it should be noted that when the polymer is under stress, the activation energy of H-abstraction from the β-methylene is lowered.[14] An attack on α-methylene may result in ester, carboxylic acid, or aldehyde groups, while an attack on β-methylene may result in alcohol, ketone, or alkene. At this point, the formation of some of these functional

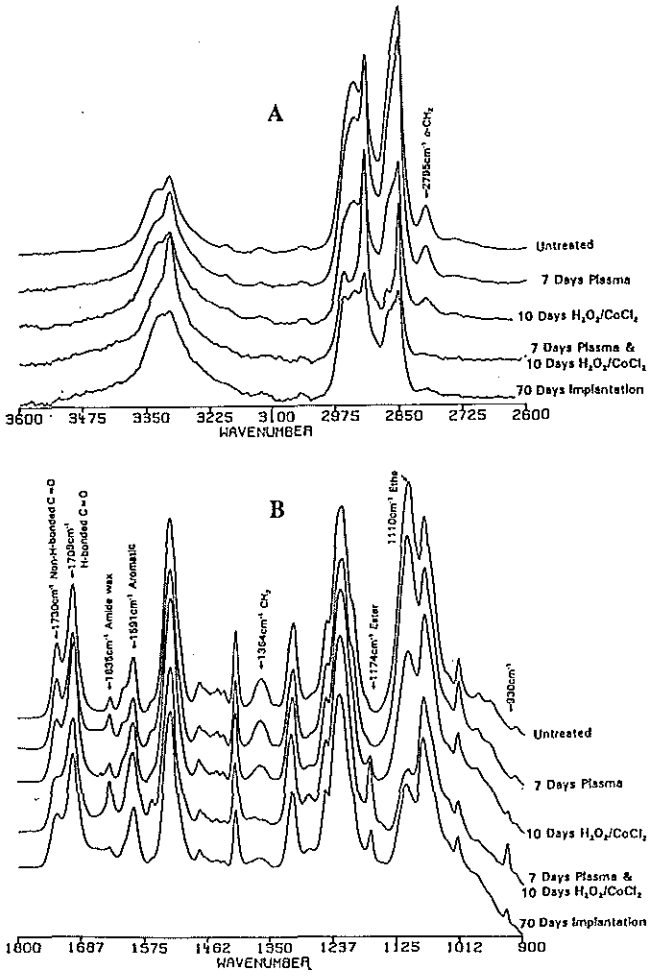

Figure 4. ATR-IR spectra of prestressed specimens before and after different treatments: Untreated; 7-day plasma treated; 7-day peroxide treated; 7-day plasma/9-day peroxide treated; 70 days (10 weeks) implanted.

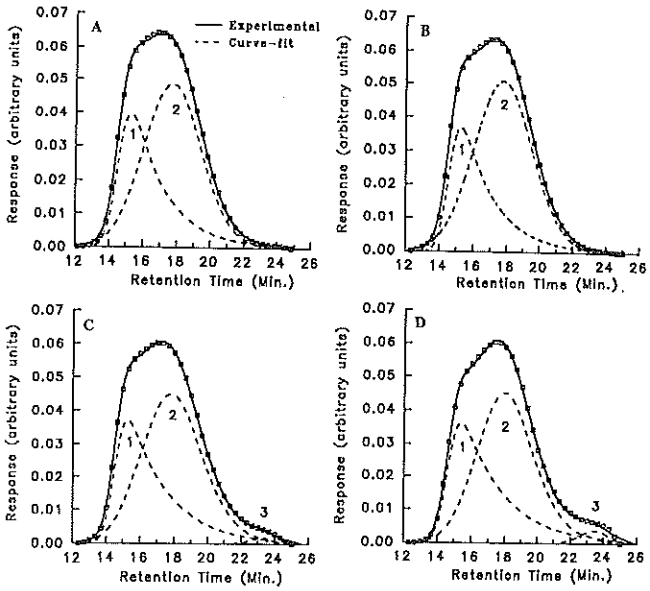

Figure 5. Experimental and deconvoluted GPC chromatograms of prestressed specimens before and after different treatments: (A) Untreated; (B) 7-day plasma treated; (C) 7-day peroxide/cobalt treated; (D) 7-day plasma plus 9-day peroxide/cobalt treated.

groups is strongly supported by comparison of the observed characteristic bands with those of model compounds. For example, the observed acid and ester groups have bands which may be correlated with those of model compounds as shown in Table III. On the other hand, some functional groups, such as aldehyde and ketone, cannot be confirmed spectrally. This means that their formation is either less likely, or that they are less stable, and after their formation they are rapidly oxidized to acid or ester groups by oxygen radicals.

Quantitative comparison of the extent of degradation between different samples can be achieved using depth profile analysis,[15] in which the concentration profile is assumed to change exponentially from surface to the bulk given by $C(x)/C_\infty = (1 - e^{-\delta x})$,

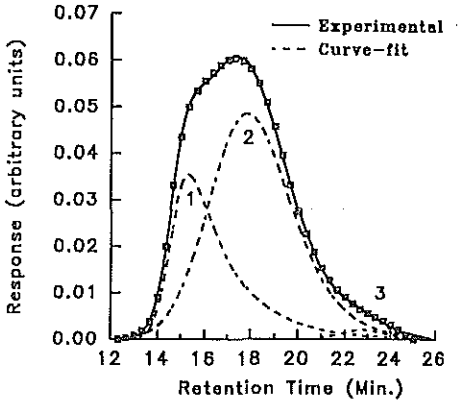

Figure 6. Experimental and deconvoluted GPC chromatograms of a prestressed specimen implanted for 70 days (10 weeks) in a Sprague-Dawley rat in the Cage Implant System.

TABLE IV
Relative Percentage of the Peaks in the GPC Chromatograms Based on the Total Amount of Polymer[a]

Sample	% Peak 1	% Peak 2	% Peak 3_b
Untreated	37.97	62.03	—
7-Day plasma	33.55	66.45	—
10-Day oxidation	38.43	60.70	0.87
7-Day plasma + 10-day oxidation	38.80	59.04	2.16
70-Day *in vivo*	32.97	65.62	1.41

[a]Peak 1 at 15.2 min, Peak 2 at 18.0 min, Peak 3 from 23.0–23.5 min.
[b]Percentage of degradation products.

where C_∞ represents the concentration in the bulk, and δ reflects the concentration change in the depth direction obtainable from the experimental data. A characteristic depth of degradation, Δ, can thus be defined as the depth at which the relative concentration becomes $(1 - e^{-1})$, or $\Delta = 1/\delta$. Since the degradation has been shown to initiate at the surface, the larger the Δ value, the greater the degree of degradation. Table VI lists the Δ values of α-methylene group from the band at 2795 cm^{-1} for samples treated under different conditions. The samples treated with plasma plus H_2O_2 (Co) had the greatest depth of degradation, which was about six times greater than that of the samples treated with H_2O_2 (Co) alone, and about 1.5 times greater than that of the *in vivo* samples. The plasma treated samples showed no degradation effect since the Δ value was about the same as the untreated samples. Thus, it is clear that plasma acts as a catalyst which accelerates the oxidative degradation of the prestressed Pellethane 2363-80A.

The GPC results demonstrated that the bulk samples, when treated with H_2O_2 (Co) or plasma plus H_2O_2 (Co), underwent a degradative process. The presence of two major peaks in the GPC chromatograms of bulk Pellethane 2363-80A indicated that the polymer contained a bimodal molecular weight distribution. For the untreated samples, the first GPC peak had an M_n of 95,000 and the second peak an M_n of 86,000 relative to polystyrene. After the samples were oxidized for 10 days in H_2O_2 (Co), the first and second GPC peaks decreased in molecular weight and a new peak appeared with an M_n of 3600 relative to polystyrene. Seventy days of implantation caused similar GPC changes in the prestressed specimens. The third peak could result from degradation products. As compared to the 10-day oxidized samples, synergistic effects of plasma plus oxidation caused a further decrease in molecular weights of the first and second peaks and a greater increase in area ratio of the third GPC peak.

Identification of biological stress cracking agents

The fact that the *in vitro* stress cracking of the Pellethane polyetherurethane requires a pretreatment with human plasma prior to H_2O_2 (Co) is intriguing. The effect of plasma plus H_2O_2 (Co) is significantly greater than the effects of plasma alone or H_2O_2 (Co) alone or the additive effects. This suggests that

TABLE V
Comparison of the Molecular Weights and Molecular Weight Distributions of the Prestressed Pellethane 80A Specimens After *In Vivo* and *In Vitro* Treatments[a]

Sample	Peak	M_n	M_w	M_z	PDI[b]
Untreated	1	9.51×10^4	5.52×10^6	9.74×10^5	5.80
	2	8.63×10^4	2.78×10^5	1.05×10^6	3.22
7-Day plasma	1	9.48×10^4	5.41×10^5	9.16×10^5	5.71
	2	8.25×10^4	2.78×10^5	1.07×10^6	3.37
10-Day oxidation	1	6.48×10^4	5.13×10^5	9.41×10^5	7.93
	2	5.90×10^4	2.52×10^5	9.43×10^5	4.27
	3	3.66×10^3	4.87×10^3	6.28×10^3	1.33
7-Day plasma + 10-Day oxidation	1	5.10×10^4	4.63×10^5	8.75×10^5	9.02
	2	5.46×10^4	2.11×10^5	6.94×10^5	3.86
	3	3.21×10^3	4.85×10^3	6.99×10^3	1.51
70-Day *in vivo*	1	7.30×10^4	5.15×10^5	9.23×10^5	7.05
	2	5.70×10^4	2.39×10^5	8.79×10^5	4.21
	3	4.76×10^3	8.13×10^3	1.20×10^4	1.71

[a]Relative to polystyrene, UV detector.
[b]Polydispersity index.

the role of plasma protein is synergistic with the role of the oxidation to produce ESC. It has been hypothesized that certain biological agents may be involved in the M_n of 86,000 relative to polystyrene. After the samples were oxidized for 10 days in H_2O_2 (Co), the first and second GPC peaks decreased in molecular weight and a new peak appeared with a M_n of 3600 relative to polystyrene. Seventy days of implantation caused similar GPC changes in the prestressed specimens. The third peak could result from degradation products. As compared to the 10-day oxidized samples, synergistic effects of plasma plus oxidation caused a further decrease in molecular weights of the first and second peaks and a greater increase in area ratio of the third GPC peak.

Identification of biological stress cracking agents

It is interesting that the in vitro stress cracking of the Pellethane polyetherurethane requires a pretreatment with human plasma prior to H_2O_2 (Co). The effect of plasma plus H_2O_2 (Co) is significantly greater than the effects of plasma alone or H_2O_2 (Co) alone or the additive effects. This suggests that the role of plasma protein is synergistic with the role of the oxidation to produce ESC. It has been hypothesized that certain biological agents may be involved in the stress cracking of polyetherurethane pacemaker insulation during *in vivo* use.[8,13] We have shown that the *in vivo* stress cracking of prestressed Pellethane specimens is dependent on viable cell/polymer interactions.[7] Adherent macrophages and foreign-body giant cells on a polyetherurethane urea film *in vivo* can produce oxidative degradation and surface cracking or embrittlement on the material at the cell/polymer contact zones.[16,17] Based on these findings, our *in vitro* system is designed to mimic the *in vivo* system; human plasma contains certain biological components that can act as a stress cracking promoter, while H_2O_2 (Co) solution provides an oxidative reaction comparable to that observed in the respiratory burst of adherent macrophages and foreign-body giant cells.[18]

To identify the biological component(s) in the *in vitro* stress cracking, the plasma was fractionated into three fractions. It was observed that a pretreatment of the stressed specimens with fraction II caused significant surface cracking where the cracking pattern [Fig. 2(B1), (B2)] resembled the characteristics of *in vivo* stress cracking. Fraction II was dominated by α_2-macroglobulin (α_2M). To test whether α_2M and/or other proteins could cause a similar effect, commercially obtained purified proteins were reconstituted to physiological concentration and employed to pretreat the prestressed specimens (Table I). Among the selected proteins, pretreatment with α_2M produced the most significant synergistic stress cracking effect [Fig. 3 (A1), (A2)] and pretreatment with ceruloplasmin a moderate effect [Fig. 3(B1), (B2)], whereas pretreatment with low-density lipoprotein or transferrin did not promote crack propagation to the brittle cracks cause by H_2O_2 (Co) oxidation [Fig. 3(C1), (C2)].

Adsorption of human plasma α_2M onto the polyetherurethane was confirmed by SDS-PAGE and immunoblotting. Human plasma α_2-macroglobulin is a large glycoprotein with four identical subunits of about 185,000 daltons each assembled pairwise by disulfide-bridges.[19] In reducing conditions, SDS-PAGE analysis revealed a protein band migrated slightly ahead of 200,000 dalton molecular weight standard (indicated by the asterisk) in Figure 7. In addition, it was observed that purified α_2M migrated to the same position. Immunoblotting with α_2M specific antibody confirmed the identity of this band to be α_2-macroglobulin.

CONCLUSIONS

This study demonstrates that the phenomenon of *in vivo* stress cracking in Pellethane 2363-80A is duplicated by an *in vitro* system that involves a pretreatment of the prestressed specimens with human plasma at 37°C for 7 days followed by oxidation in 10% hydrogen peroxide with 0.10*M* cobalt chloride at 50°C for 10 days. Morphologically, the pattern of *in vitro* stress cracking has remarkable similarity to that of *in vivo* stress cracking with characteristics of ductile failure. Chemical analysis by ATR-FTIR and GPC indicates that *in vitro* and *in vivo* treated samples are degraded by an oxidative reaction that takes place mainly in the polyether soft segments with oxygen

TABLE VI
ATR-FTIR Analysis of Treated and Untreated Pellethane 2363-80A: Parameters of Degradation Depths for α-Methylene Group at 2795 cm^{-1}

Parameters	Untreated	Treated with Plasma	Treated with H_2O_2 (Co)	Treated with Plasma Plus H_2O_2 (Co)	Implanted for 70 days
K	0.53	0.59	0.57	0.67	—
δ (μm^{-1})	6.1	5.5	2.1	0.32	0.53
Δ (μm)	0.17	0.18	0.47	3.1	1.9
Δ_{Tr}/Δ_{Un}	1.0	1.1	2.8	18.8	11.2

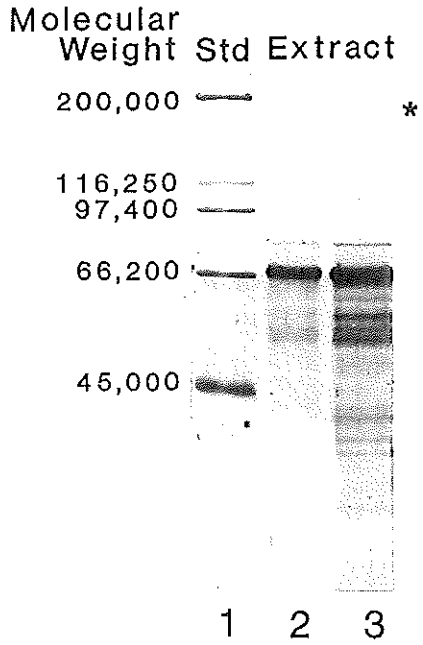

Figure 7. Photograph of SDS-PAGE bands of adsorbed proteins on Pellethane 2363-80A. The protein band indicated by the asterisk is confirmed to be α_2-Macroglobulin by immunoblotting.

radicals attacking α- and β-methylene groups. *In vitro* pretreatment with plasma has a synergistic effect with the oxidation facilitated by H_2O_2 (Co) treatment to produce ESC.

A plasma component responsible for promoting stress cracking in Pellethane 2363-80A polyurethane is identified to be α_2-macroglobulin (α_2M). Pretreatment with α_2M-rich plasma fraction or commercially obtained purified protein solution has a significant stress cracking effect on the prestressed specimens. Adsorption of human plasma α_2M onto the polymer is confirmed by SDS-PAGE and immunoblotting.

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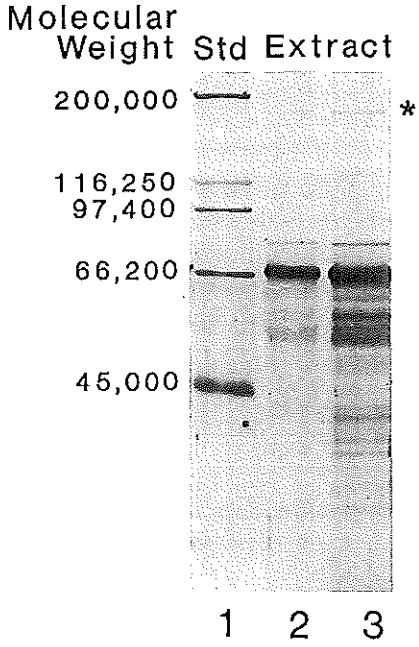

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